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Is There a Critical Target Gene for the First Step in Carcinogenesis?

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Our work has suggested that a high-frequency event is involved in the initiation phase of malignant transformation *in vitro*; a later, mutationlike event appears to be involved in the later stages of transformation. There may be no specific "target gene" which directly interacts with carcinogens. It is hypothesized that nonspecific types of DNA damage are involved in the induction of an ongoing process we know as carcinogenesis. Several genes could be involved in maintaining this process. Our recent results suggest that c-*myc* and c-*fos* could be involved in the early stages of carcinogenesis, as they are affected by anticarcinogenic protease inhibitors in a manner that corresponds to the way in which protease inhibitors suppress malignant transformation.

Nature of the Initiating Event in Carcinogenesis

Our previous work has suggested that a high-frequency event is involved in the induction of radiation-induced transformation *in vitro* (*1–8*). The work of several other investigators has now suggested that a similar high-frequency initiating event occurs in carcinogenesis in both *in vitro* and *in vivo* systems, with many different types of DNA-damaging agents initiating the carcinogenic process, as has been reviewed elsewhere (*7–11*).

Given the high-frequency nature of the initiating event in carcinogenesis, it is unlikely to be a specific locus mutation, as studies of mutation frequencies have shown them to occur at orders of magnitude below those observed for malignant transformation. The initiating event does not behave like a mutation, as it appears to be a reversible phenomenon. We have observed that certain protease inhibitors, which are highly effective in their ability to suppress malignant transformation *in vitro* (*12*) and *in vivo* (*13*), are capable of reversing initiation (*14*). There is much evidence from *in vivo* studies that lesions thought to represent "initiated" or "premalignant" cells are capable of reverting to their normal state. For example, Terzaghi-Howe (*15*) observed that contact with normal tracheal epithelium could revert initiated "pre-neoplastic" tracheal epithelial cells to a normal condition. It is well known that "premalignant" lesions *in vivo*, such as squamous metaplasia, dysplasia, etc., are readily reversible in nature.

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The nature of the high-frequency initiating event is unknown. Several radiation/carcinogen-induced processes that could be involved in carcinogenesis and are likely explanations for our observations have been discussed elsewhere (*1–8*). There are many possibilities for the initiating event in carcinogenesis. For example, carcinogens such as radiation have been shown to alter DNA methylation patterns in a widespread fashion (*16*). Methylation of DNA is thought to play an important role in gene regulation; we have hypothesized that the initiating event in carcinogenesis involves altered gene expression (*1–8*). Another event that is induced in a widespread fashion in a population of mammalian cells by a number of different carcinogens (including radiation) is gene amplification (*17–19*). It is of interest to us that modifiers of carcinogenesis also affect the level of gene amplification; for example, gene amplification can be potentiated by tumor-promoting agents such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (*19*) and inhibited by agents that suppress carcinogenesis, such as certain protease inhibitors known to have anticarcinogenic activity (*20*). The nature of the initiating event in carcinogenesis deserves much more intensive study.

Target Genes in Carcinogenesis

Several genes are likely to be involved in maintenance of the ongoing process induced by carcinogens. Two genes are of particular interest to us in that their expression is affected by the anticarcinogenic protease inhibitors in a manner which corresponds to the way in which the protease inhibitors affect the induction of transformation *in vitro*; these genes are c-*myc* (*21–24*) and c-*fos* (*25*). Protease inhibitors affect the expression of these genes as summarized in Table 1. As the anti-

Table 1. Suppression of gene expression by protease inhibitors.[a]

Protease inhibitor	Ability of protease inhibitors to suppress: Actin expression	c-*myc* expression: Nontransformed cells	c-*myc* expression: Transformed cells	c-*fos* expression	Radiation-induced transformation
Bowman-Birk	–	+ +	–	+ + +	+ + +
Antipain	–	+ + +	–	+ +	+ +
Leupeptin	ND[b]	+ +	ND	ND	+ +
α_1-antitrypsin	ND	–	ND	–	–
Elastatinal	ND	–	ND	ND	–
Soybean trypsin inhibitor	ND	–	ND	ND	–

[a]In terms of effective molar concentrations of protease inhibitors.
[b]ND, not determined.

carcinogenic protease inhibitors are not capable of affecting c-*myc* expression in transformed cells but do affect c-*myc* in nontransformed cells, our results suggest that c-*myc* regulation may be of great importance in the malignant transformation of cells, as discussed in detail elsewhere (*24*).

We have hypothesized that the anticarcinogenic protease inhibitor effects on c-*myc* expression and malignant transformation are involved in an early stage of carcinogenesis, even though protease inhibitors can affect carcinogenesis at long time periods after carcinogen exposure (*14*). Our proposed scheme for the induction of malignant transformation *in vitro* is shown in Figure 1.

Carcinogens such as radiation are known to induce c-*myc*; we have shown that c-*myc* is induced *in vivo* by radiation and that anticarcinogenic protease inhibitors reduce c-*myc* expression to normal levels (*26*). It has also been shown that c-*myc* expression is elevated in radiation-induced tumors (*27*). As shown in Figure 1, it is expected that anticarcinogenic protease inhibitors reduce c-*myc* expression to normal levels after carcinogen exposure; this phenomenon has been shown to occur in the irradiated mouse colon (*26*). It is possible that elevated c-*myc* expression influences the level of expression of another gene—specifically, as shown in Figure 1, a gene coding for a particular protease, the Boc-Val-Pro-Arg-MCA hydrolyzing activity, which has been studied extensively in our laboratory (*13,28–30*).

The c-*myc* gene codes for a nuclear binding protein and is thought to play a regulatory role in gene transcription (*31*). Our research would suggest that there must be persistent activation of the process involved in malignant transformation. While c-*myc* is only transiently activated by radiation (*26*), the Boc-Val-Pro-Arg-MCA hydrolyzing activity is persistently activated by carcinogen exposure (*30*). We have observed higher than normal levels of Boc-Val-Pro-Arg-MCA hydrolyzing activity in normal-appearing areas of carcinogen-treated epithelial cells *in vivo*, even at long time periods after carcinogen exposure (*30*). This proteolytic activity is directly affected by the anticarcinogenic protease inhibitors in a manner that corresponds to the way in which these agents suppress malignant transformation *in vitro* (*13,28*) and *in vivo* (*13,30*). As shown in Figure 1, it is proposed that c-*myc* induction precedes the induction of the protease (Boc-Val-Pro-Arg-MCA hydrolyzing activity). It is perhaps equally likely that the order of these two phenomena is reversed, as it is known that proteases such as plasminogen activator induce c-*myc* expression. Many other agents studied in carcinogenesis research induce c-*myc* expression; for example, TPA is known to induce c-*myc* gene expression (*32*).

We have hypothesized that a late event is involved

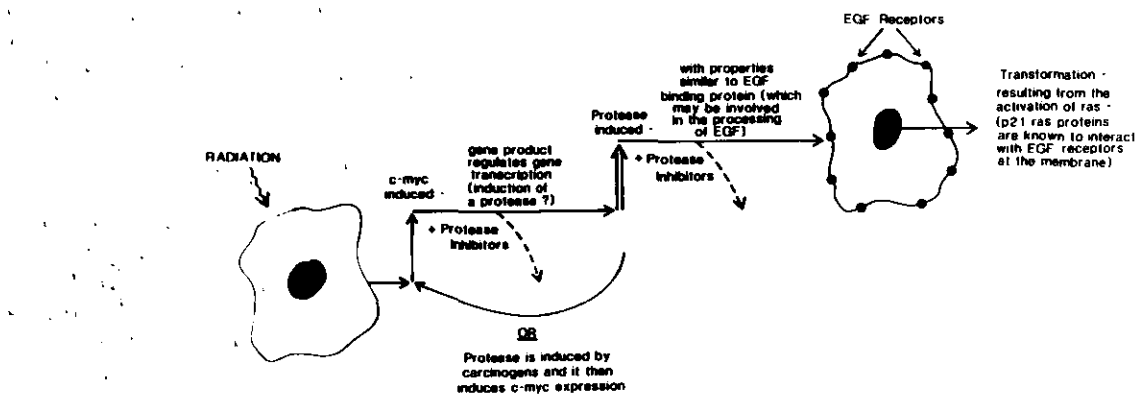


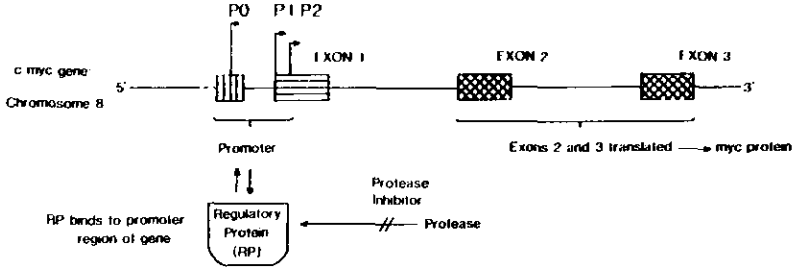
FIGURE 1. Our hypothesized scheme for events involved in radiation transformation *in vitro*. Anticarcinogenic protease inhibitors have been shown to affect both c-*myc* and a specific proteolytic activity (the Boc-Val-Pro-Arg-MCA hydrolyzing activity), as described in the text.

in actually transforming a cell to the malignant state (*1–8,12*). In Figure 1, we have hypothesized that the activation of *ras* is involved in a late stage of carcinogenesis. There are several ways in which an interaction between the events we believe are related to the early stages of carcinogenesis (i.e., the induction of c-*myc* and the induction of a protease such as the Boc-Val-Pro-Arg-MCA hydrolyzing activity) and members of the *ras* gene family could occur. Cooperation between *myc* and *ras* in the induction of transformation is well documented (*33,34*). The Boc-Val-Pro-Arg-MCA hydrolyzing activity has characteristics that make it likely to be involved in the processing of a growth factor like epidermal growth factor (EGF) (*13,28*). If this proteolytic activity were involved in the processing of a growth factor like EGF, more of an EGF-like substance would be present than under normal conditions. Carcinogen-treated cells growing under the influence of abnormally large amounts of a growth factor would be likely to exhibit altered growth characteristics; such altered growth patterns are known to occur in a widespread fashion in carcinogen-treated tissue (and are known as premalignant changes). We propose that an additional change occurs in these atypical areas and that it is this later change that leads directly to malignancy; the evidence for such a late step in the malignant transformation of C3H10T1/2 cells and carcinogenesis *in vivo* has been reviewed (*1–8,12*). We propose here that this later change involves the activation of *ras*, which occurs as a late event in several *in vitro* systems (e.g., *35*) and is known to be activated in many different kinds of cancers, including those induced by radiation (*36,37*). It is possible that the activation of *ras* is connected to the cellular effects brought about by EGF, as it is known that the p21 proteins of *ras* interact with the EGF receptor (the product of c-Ha-*ras* is activated by EGF) (*38*). Although the Boc-Val-Pro-Arg-MCA hydrolyzing activity we have studied has characteristics similar to EGF binding protein (*28*), which is thought to be involved in the processing of EGF, it is not exactly like EGF binding protein. Thus, we have hypothesized that a growth factor involved in the malignant transformation of cells in the systems we have used may be similar to EGF.

There is some evidence to suggest that a growth factor like EGF is involved in the induction of transformation *in vitro*; for example, EGF is known to promote transformation *in vitro* (*39*), and it is known that EGF as a promoting agent can bring about an irreversible change in cells (such as a point mutation in *ras* that leads to its activation), that is, the switch to anchorage-independent growth (which correlates with tumorigenicity) in promotable cells (*40*).

Protease Inhibitor Suppression of c-*myc* Gene Expression

The mechanism by which anticarcinogenic protease inhibitors suppress c-*myc* gene expression is unknown, although many hypotheses have been presented elsewhere (*21–24*). A potential model for c-*myc* gene expression and its regulation by a protease is shown in Figure 2. Our model proposes that a protease is capable of destroying a regulatory protein involved in the regulation of c-*myc*; this regulatory protein would conceivably bind to the promoter region of the gene, as shown in Figure 2. Carcinogens could increase the level of the protease, which would lead to decreased levels of the regulatory protein; decreased binding of the regulatory



MODEL FOR RADIATION AND PROTEASE INHIBITOR EFFECTS ON c-myc EXPRESSION

	PROTEASE LEVELS	REGULATORY PROTEIN (RP)	LEVEL OF EXPRESSION OF c-myc
Normal regulation of c-myc gene	↓	On - RP bound to promoter	↓
	↑	Off - RP not bound to promoter	↑
Radiation	↑↑[2]	RP destroyed by protease, RP not bound to promoter	↑[1]
Radiation & Anticarcinogenic protease inhibitors	↓[3]	RP no longer being destroyed by increased protease levels, thus RP can bind to promoter	↓

FIGURE 2. Proposed model to explain c-*myc* gene expression and its regulation by a protease. It is hypothesized that anticarcinogenic protease inhibitors operate in our proposed model as described in the text. (1–3) Evidence in support of proposed model: c-*myc* expression increases in radiation-induced tumors *in vivo* protease levels increase in carcinogen-treated tissue *in vivo* (*30*), anticarcinogenic protease inhibitors return protease levels to normal (*30*).

protein to the promoter region of c-*myc* would then lead to increased levels of c-*myc* gene expression. Evidence in support of this part of the proposed model comes from experiments showing that: *a*) carcinogens induce elevated levels of a protease: Boc-Val-Pro-Arg-MCA hydrolyzing activity (*13,28*); *b*) radiation increases c-*myc* gene expression (*26*); and c) c-myc gene expression increases in radiation-induced tumors *in vivo* (*27*).

Conceivably, anticarcinogenic protease inhibitors could then inhibit the protease that destroys the regulatory protein. In fact, anticarcinogenic protease inhibitors have been shown to inhibit carcinogen-induced protease activity, the Boc-Val-Pro-Arg-MCA hydrolyzing activity, *in vivo* (*30*), and *in vitro* (*13,28*), as well as radiation-induced c-*myc* levels *in vivo* (*26*).

We are currently attempting to determine whether the schematic presentation in Figure 2 is actually occurring during the regulation of c-*myc* expression by protease inhibitors. Current hypotheses for the mechanism of regulation of the c-*myc* gene are described in detail elsewhere (*23,24*). While the exact characteristics of the regulatory protein hypothesized to be involved in c-*myc* gene regulation are unknown, Zajac-Kaye et al. (*41*) have described a DNA-binding activity that binds to the 5′ region of the first intron of c-*myc*; this binding activity is thought to be intimately involved in c-*myc* gene regulation. We are performing experiments to determine whether alterations in the levels or other changes in this DNA-binding activity can explain our observations on protease inhibitors and c-*myc* gene expression as they relate to carcinogenesis.

Although the models of carcinogenesis presented here are highly speculative, the anticarcinogenic protease inhibitor effects on the suppression of c-*myc* and c-*fos* gene expression and the Boc-Val-Pro-Arg-MCA hydrolyzing activity are well documented. It is believed that the effects of those agents that modify carcinogenesis on specific genes may lead us to an understanding of the role these genes play in the carcinogenic process.

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REFERENCES

1. Kennedy, A. R., Fox, M., Murphy, G., and Little, J. B. Relationship between x-ray exposure and malignant transformation in C3H 101/2 cells. Proc. Natl. Acad. Sci. U.S.A. 77: 7262–7266 (1990).
2. Kennedy, A. R., and Little, J. B. An investigation of the mechanism for the enhancement of radiation transformation *in vitro* by TPA. Carcinogenesis 1: 1039–1047 (1980).
3. Kennedy, A. R., and Little, J. B. High efficiency, kinetics and numerology of transformation *in vitro*. In: Cancer: Achievements, Challenges and Prospects for the 1980s, Vol. 1 (J.H. Burchenal and J.F. Oettgen, Ed.), Grune and Stratton Inc., Philadelphia, 1981, pp. 491–500.
4. Kennedy, A. R. Promotion and other interactions between agents in the induction of transformation *in vitro* in fibroblasts. In: Mechanisms of Tumor Promotion, Vol. III, Tumor Promotion and Carcinogenesis *In Vitro* (T.J. Slaga, Ed.), CRC Press, Inc., Boca Raton, FL, 1984, pp. 13–55.
5. Kennedy, A. R., Cairns, J., and Little, J. B. The timing of the steps in transformation of C3H10T1/2 cells by X-irradiation. Nature 307: 85–86 (1984).
6. Kennedy, A. R., and Little, J. B. Evidence that a second event in x-ray induced oncogenic transformation *in vitro* occurs during cellular proliferation. Radiation Res. 99: 228–248 (1984).
7. Kennedy, A. R. Evidence that the first step leading to carcinogen-induced malignant transformation is a high frequency, common event. In: Carcinogenesis; A Comprehensive Survey: Mammalian Cell Transformation: Mechanisms of Carcinogenesis and Assays for Carcinogens, Vol. 9 (J.C. Barrett and R.W. Tennant, Eds.), Raven Press, New York, 1985, pp. 355–364.
8. Kennedy, A. R. Initiation and promotion of radiation induced transformation *in vitro*: relevance of *in vitro* studies to radiation induced cancer in human populations. In: Cell Transformation Systems Relevant to Radiation-induced Cancer in Man (K.H. Chadwick, Ed.), IOP Publishing, Ltd, Bristol, England, 1989, pp. 263–270.
9. Clifton, K. H. The clonogenic cells of the mammary and thyroid glands: their biology, frequency of initiation and promotion/progression to cancer. In: Mathematical Modeling: Statistical Issues in Cancer Risk Assessment (S. Moolgavkar and D. Thomsen, Eds.), 1989, Birkhouser, Inc., Boston, in press.
10. Watanabe, H., Tanner, M. A., Domann, F. E., Gould, M. N., and Clifton, K. H. Inhibition of carcinoma formation and of vascular invasion in grafts of radiation-initiated thyroid clonogens by unirradiated thyroid cells. Carcinogenesis 9: 1329–1335 (1988).
11. Clifton, K. H., Tanner, M. A., and Gould, M. N. Assessment of radiogenic cancer initiation frequency per clonogenic rat mammary cell *in vivo*. Cancer Res. 46: 2390–2395 (1986).
12. Kennedy, A. R. Implications for mechanisms of tumor promotion and its inhibition by various agents from studies of *in vitro* transformation. In: Tumor Promoters, Biological Approaches for Mechanistic Studies and Assay Systems (R. Langenbach, J.C. Barrett, and E. Elmore, Eds.), Raven Press, New York, 1988, pp. 201–212.
13. Kennedy, A. R., and Billings, P. C. Anticarcinogenic actions of protease inhibitors. In: Anticarcinogenesis and Radiation Protection (P.A. Cerrutti, O.F. Nygaard, and M.G. Simic, Eds.), Plenum Press, New York, 1987, pp. 285–295.
14. Kennedy, A. R. The conditions for the modification of radiation transformation *in vitro* by a tumor promoter and protease inhibitors. Carcinogenesis 6: 1441–1446 (1985).
15. Terzaghi-Howe, M. Inhibition of carcinogen-altered rat tracheal epithelial cell proliferation by normal epithelial cells *in vivo*. Carcinogenesis 8: 145–150 (1987).
16. Kalinich, J. F., Catravas, G. N., and Snyder, S. L. The effect of gamma radiation on DNA methylation. Radiat. Res. 117: 185–197 (1989).
17. Lavi, S. Carcinogen-mediated amplification of viral DNA sequences in simian virus 40-transformed Chinese hamster embryo cells. Proc. Natl. Acad. Sci. U.S.A. 78: 6144–6148 (1981).
18. Lavi, S. Carcinogen-mediated amplification of specific DNA sequences. J. Cell Biochem. 18: 149–156 (1986).
19. Tlsty, T. O., Brown, P. C., and Schimke, R. T. Ultraviolet radiation facilitates methotrexate resistance and amplification of the dihydrofolate reductase gene in cultured 3T6 mouse cells. Mol. Cell. Biol. 4: 1050–1056 (1984).
20. Heilbronn, R., Schlehofer, J. R., Yalkinoglu, A. O., and Zur Hausen, H. Selective DNA amplification induced by carcinogens (initiators): evidence for a role of proteases and DNA polymerase alpha. Int. J. Cancer 36: 85–91 (1985).
21. Chang, J. D., Billings, P., and Kennedy, A. R. C-myc expression is reduced in antipain-treated proliferating C3H10T1/2 cells. Biochem. Biophys. Res. Commun. 133: 830–835 (1985).
22. Chang, J. D., and Kennedy, A. R. Cell cycle progression of C3H10T1/2 and 3T3 cells in the absence of a transient increase in c-myc RNA levels. Carcinogenesis 9: 17–20 (1988).
23. Chang, J. D., Li, J.-H., Billings, P. C., and Kennedy, A. R. Effects of protease inhibitors on c-myc expression in normal and transformed C3H10T1/2 cells. Mol. Carcinog. 3: 226–232 (1990).
24. Chang, J. D., and Kennedy, A. R. Suppression of c-myc by anticarcinogenic protease inhibitors. In: Protease Inhibitors as Can-

cer Chemopreventive Agents (W. Troll and A. Kennedy, Eds.), Plenum Publishing Corporation, New York, in press.
25. Caggana, M., and Kennedy, A. R. C-fos mRNA levels are reduced in the presence of antipain and the Bowman-Birk inhibitor. Carcinogenesis 10: 2145–2148 (1989).
26. St. Clair, W. H., Billings, P. C., and Kennedy, A. R. The effects of the Bowman-Birk protease inhibitor on c-myc expression and cell proliferation in the unirradiated and irradiated mouse colon. Cancer Lett. 52: 145–152 (1990).
27. Sawey, M. J., Hood, A. T., Burns, F. J., and Garte, S. J. Activation of myc and ras oncogenes in primary rat tumors induced by ionizing radiation. Mol. Cell. Biol. 7: 932–935 (1987).
28. Billings, P. C., Carew, J. A., Keller-McGandy, C. E., Goldberg, A., and Kennedy, A. R. A serine protease activity in C3H/10T1/2 cells that is inhibited by anticarcinogenic protease inhibitors. Proc. Natl. Acad. Sci. U.S.A. 84: 4801–4805 (1987).
29. *Billings, P. C., St. Clair, W., Owen, A. J., and Kennedy, A. R.* Potential intracellular target proteins of the anticarcinogenic Bowman-Birk protease inhibitor identified by affinity chromatography. Cancer Res. 48: 1798–1802 (1988).
30. Messadi, P. V., Billings, P., Shklar, G., and Kennedy, A. R. Inhibition of oral carcinogenesis by a protease inhibitor. J. Natl. Cancer Inst. 76: 447–452 (1986).
31. Bishop, J. M. Viral oncogenes. Cell 42: 23–38 (1985).
32. Kelly, K., Cochran, B. H., Stiles, C. D., and Leder, P. Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. Cell 35: 603–610 (1983).
33. Land, H., Parada, L. F., and Weinberg, R. A. Cellular oncogenes and multistep carcinogenesis. Science 222: 771–778 (1983).
34. Land, H., Parada, L. F., and Weinberg, R. A. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 304: 596–602 (1983).
35. Sukumar, S., Pulciani, S., Doniger, J., DiPaolo, J. A., Evans, C. H., Zbar, B., and Barbacid, M. A transforming ras gene in tumorigenic guinea pig cell lines initiated by diverse chemical carcinogens. Science 223: 1197–1199 (1985).
36. Guerrero, I., Calzada, P., Mayer, A., and Pellicer, A. A molecular approach to leukemogenesis: mouse lymphomas contain an activated c-ras oncogene. Proc. Natl. Acad. Sci. U.S.A. 81: 202–205 (1984).
37. Frazier, M. E., Lindberg, R. A., Mueller, D. M., Gee, A., and Seed, T. M. Oncogene involvement in plutonium-induced carcinogenesis. Int. J. Radiat. Biol. 49: 542–543 (1986).
38. Kamata, T., and Feramisco, T. R. Epidermal growth factor stimulates guanine nucleotide binding activity and phosphorylation of ras oncogenes. Nature 310: 147 (1984).
39. Little, J. B., and Kennedy, A. R. Promotion of X-ray transformation *in vitro*. In: Carcinogenesis, Vol. 7 (E. Hecker, N. E. Fusenig, W. Kunz, F. Marks, and H. W. Thielmann, Eds.), Raven Press, New York, 1982, pp. 243–257.
40. Colburn, N. H., and Gindhart, T. D. Specific binding of transforming growth factor correlates with promotion of anchorage independence in mouse JB6 cells. Biochem. Biophys. Res. Commun. 102: 799–807 (1981).
41. Zajac-Kaye, M., Gelmann, E. P., and Levens, D. A point mutation in the c-myc locus of a Burkitt lymphoma abolishes binding of a nuclear protein. Science 240: 1776–1779 (1988).